Comm.
Dr. Jacobson
Dr. Loosli
Dr. Wyatt

PULMONARY

The Council For Tobacco Research–U.S.A., Inc.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JUL 27 1973

Application for Research Grant
(Use extra pages as needed)

Date: 7/17/73

1. Principal Investigator (give title and degrees):

Daniel B. Rifkin, Assistant Professor of Chemical Biology, Ph.D.

2. Institution & address:

The Rockefeller University
New York, New York 10021

3. Department(s) where research will be done or collaboration provided:

Department of Chemical Biology

4. Short title of study:

Proteases Produced by Mammalian Lung Tissue

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

The aim of this project is the characterization of a low molecular weight protease produced by human embryonic lung cells in vitro. This protease is a potent activator of serum proenzymes and may be involved in certain pathological conditions of the human lung, namely respiratory distress syndrome (RDS) and chronic emphysema. The proteases from human and mouse lung cells grown in culture will be purified and characterized as to their physical properties, mode of action, substrate specificity, and ability to activate proenzymes such as plasminogen procollagenase and pro-elastase. Antibodies will be made to the purified proteins. These antibodies will be used to determine at what stage in the embryonic development of the mammalian lung this enzyme appears and if it continues to be produced after parturition. Fluorescein labeled antibody will be employed as an emmunofluorescent probe to test if other cells in preparations of fixed lung tissue are producing this enzyme. Finally, experiments will be designed to ascertain whether the production of the lung enzyme can be influenced by drugs such as corticosteriods.

It is hoped that this analysis will provide information necessary to determine if this protease may be involved in the respiratory disease, and, if so, how one may develop therapeutic approaches to these conditions.

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8. Brief statement of working hypothesis:

(see attached)

9. Details of experimental design and procedures (append extra pages as necessary)

(see attached)

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8.

We have recently reported that many neoplastic cells produce a proteolytic enzyme while their normal counterparts do not (1, 2). In the course of a study of transformed and normal human cells, it was found that normal human embryonic lung cells (fibroblasts) produce an enzyme which is similar in many of its characteristics to the enzyme produced by human tumor cells (3). This preliminary observation has been extended to cultures of human lung cells derived from four separate embryos (4). While the presence of proteolytic activities in lung are well known, they are usually attributed to leukocytes and macrophages (5-7). This is the first description of an extracellular protease produced by cells which are associated with the structural elements of the lung. It is our hypothesis that the control of production of this enzyme may be important in pathological conditions of the lung for the following reasons:

1) It has been reported that proteolytic treatment of rodent lungs produces a condition similar to that found in human chronic emphysema (8, 9). While it is not surprising that the rather harsh treatments utilized in these experiments caused tissue destruction, a source in human lungs capable of producing proteolytic activities in amounts adequate for such tissue destruction has yet to be described. The activities associated with leukocytes and macrophages are not substantial (10, 11) and may well be totally inhibited in vivo by serum inhibitors such as the α_1-antitrypsin. However, the proteolytic activity generated by the lung fibroblasts is substantial. As few as 5×10^5 cells can produce enough protease to digest their own weight in protein in 17 hours even under conditions where serum inhibitors are present (11). It appears that the proteolytic inhibitors in serum do have an effect on the protease produced by lung fibroblasts but the rate of production of enzyme is great enough to overcome this inhibition. The α_1-antitrypsin is the most effective of the serum inhibitors (12). This of interest because of the correlation of low levels of α_1-antitrypsin and the propensity towards chronic emphysema in certain individuals (13-15). Finally, preliminary experiments suggest that this enzyme also can activate proenzymes such as procollagenase and proelastase. Our hypothesis is that either the abnormal production of the lung cell protease or the failure to inhibit the protoelytic activity which is normally generated by lung fibroblasts is responsible for chronic emphysema.

2) Respiratory distress syndrome (RDS) is correlated with a histological picture of hyaline membrane formation. Clinically it has been found that infants with RDS have low plasminogen (16) and low α_1-antitrypsin (17-19) levels as well as smaller adrenal glands (20). We hypothesize that in infants with RDS there is an insufficient production of adrenal corticosteriods and therefore, a failure to inhibit the formation of the lung cell protease at birth, since it has recently been reported that corticosteroids prevent the production of proteolytic enzymes similar to the enzyme made by lung fibroblasts (21). Because of the continued production of the lung cell enzyme there is an abnormal activation of plasminogen. The plasmin thus produced is inactivated in turn by the circulating α_1-antitrypsin. This combination of events would account for the clinical picture of plasminogen and α_1-antitrypsin depletion. Moreover the lung would be unable to dissolve the hyaline membrane once formed since this structure is composed primarily of fibrin (22) and there would be little plasminogen available

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8. (continued)

for lysis. This type of hypothesis has been proposed before as an explanation of RDS (23) but the type of analysis used was extremely crude and, therefore, the results difficult to interpret in some cases. It seems worth while to reinvestigate the role of proteases, specifically the lung cell enzyme, in RDS.

With these facts in mind we propose to initiate an investigation of the role of the lung protease by first characterizing its physical and functional properties as well as the control of its synthesis using the experimental plan described.

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Initially our efforts will be directed towards the purification and characterization of the cell protease produced by both human and mouse embryonic lung cells in culture. Mouse cells will be prepared from trypsinized mouse embryonic lung, while the human cells will be isolated from the lungs of human fetuses obtained surgically. We have received assurances from physicians at Columbia-Presbyterian Hospital that it will be possible to secure the human tissue which we will need. Once these cells have been established in culture they will be checked for the production of the cell protease. This is done by incubating cells in medium lacking serum. The conditioned medium is removed, and clarified of debris by centrifugation. Aliquots of the supernatant solution are then incubated with purified plasminogen in plastic dishes which have been coated with ^{125}I-fibrin. The lung protease has been shown to activate plasminogen extremely effectively and the proteolytic activity of the plasmin formed is assessed by monitoring the release of ^{125}I from the surface of the dish. It should be noted that the activation of plasminogen in this system is accomplished by a proteolytic cleavage of the proenzyme and is, therefore, a true measure of the cell produced protease. (This assay has been published and is routinely used in our laboratory)[1].

The cell factor will then be purified from large quantities of conditioned medium. Preliminary experiments which we have performed indicate that the conditioned medium contains approximately 20 μg of enzyme per liter. Since the cells produce this enzyme continuously for several days in the absence of serum and the culture medium is saturated with active enzyme after 4-5 hours of incubation, large quantities of conditioned medium can be prepared with reasonable facility. (In a pilot experiment we have prepared 200 l of conditioned medium from human tumor cells in a three week period). The active enzyme will then be purified using the techniques of ammonium sulfate precipitation, SP-sephadex chromatography, gel chromatography, preparative acrylamide gel electrophoresis, and isoelectric focusing. These techniques have been used successfully to purify similar enzymes from transformed hamster and chicken cells (24, 25).

In this manner it should be possible to prepare milligram amounts of homogeneous enzyme. This material will then be used to characterize the physical and chemical properties of the enzyme. The molecular weight will be determined by SDS-polyacrylamide gel electrophoresis. Reactive groups in the molecule will be assayed using conventional techniques. For example, the requirement for disulfide bonds for an active enzyme will be tested by first reducing and alkylating any S-S-bonds and then testing for enzymatic activity. The type of protease will be assessed by testing proteolytic inhibitors of known activity such as DFP, TPLK, NPGB, E-ACA, TPCK (26). A number of naturally occuring inhibitors also will be screened since these may be particularly useful if this enzyme proves to be of any clinical significance. Experiments will be performed to see what molecules may act as substrates for the enzyme. A number of different substrates will be tested, such as BSA, casein, collagen, elastin, fibrin, and protamine. In addition the ability of this enzyme to activate proenzymes found in the serum or lung will be assessed. These will include proelastase, procollagenase, and prothrombin. These proenzymes will be obtained either from commercial sources or individual investigators and will be assayed by published procedures. We have determined that this enzyme can activate plasminogen extremely efficiently. Finally, attempts will be

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made to determine if this protease has any activity upon the structural elements of the lung. Basement membrane from lung alveoli will be prepared as described by Kefalides (27), made radioactive by a labeling procedure (28), and the ability of the lung enzyme to degrade basement membrane assayed by the release of soluble radioactive material from the insoluble basement membrane.

The largest portion of the purified material will be used to produce antibodies in rabbits. (If we are unable to produce antibodies in rabbits, other animals will be used such as goats or sheep). A micro method will be employed where the antigen in an adjuvent solution is injected directly into a lymph node (29). This procedure is repeated several times at two week intervals. If successful it is possible to induce antibodies with 3-4 milligrams of purified material. This is undoubtedly the most crucial part of the research plan, since antibody against the lung protease will allow us to initiate some of the biological experiments described below. We are fortunate that there are several laboratories at Rockefeller which have had a great deal of experience in the production of antibodies to purified molecules and they have promised their assistance if necessary.

Having established the fundamental properties of the enzyme and having produced antibodies to the protease, we will then attempt to study those factors which may be important in the control of the synthesis of this protein. We will first attempt to follow the production of the enzyme histologically as a function of time using anti-lung protease antibody conjugated to a fluorescent dye (30). Mouse embryos and weenlings of various ages will be secured, and the lungs isolated, fixed, stained with the fluorescent antibody, and examined microscopically. This will allow us to determine at what time the enzyme is initially produced in the lung, for how long a period it is produced, and what other cell types besides fibroblasts produce it. Control tissue such as skin or muscle will also be tested since our own preliminary experiments indicate that fibroblasts derived from skin biopsies do not produce this enzyme. These experiments are extremely important because it may well be that cells which do not demonstrate an enzymatic activity when grown in vitro contain the enzyme in an inactive precursor form or in a form which is inhibited by a second molecule. It is hoped that there precursor or inactivated molecules will be detected by an immuno assay of histological specimins.

Experiments are planned to test the effect of drugs on the production of this enzyme. Lung cells will be grown in vitro in the presence or absence of different drugs and the amount of enzyme produced assayed either by its activity or by immune precipitation (31). The types of drugs which will be tested are adrenal steroids, immunosuppressors, polypeptide hormones, and drugs which affect platelet cell functions or fibrinolysis. If any of these drugs look promising in terms of either increasing or decreasing the production of the enzyme, experiments will be initiated to test the effects of these compounds in animals. The effects will be assayed immunologically on histological preparations.

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9. (continued)

Finally it is hoped that these tools will be applied to an analysis of the human lung in RDS and chronic lung emphesema. The main objective would be to study the occurence and activity of this protein as a function of age, in relation to inhibitors and activators found in the lung, and the spatial distribution of enzyme within the tissue. This aspect of the work is at present only in the formative stage as it would require the collaboration of a number of physicians at neighboring hospitals and it is felt that it would be premature to set up such collaborations before more evidence is at hand as to the function of the lung enzyme.

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Bibliography

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20. Naeye, R.L. et al. Pediatrics 47: 650 (1971).
21. Wunachmann, Henderson, B. and Astrup, T. Fed. Proc. (1973).
22. Gitlin, D. and Craig, J.M. Pediatrics 17: 64 (1956).
23. Lieberman, J. N. Eng. J. Med. 262: 999 (1960).
24. Unkeless, J.C. et al. Manuscript submitted to J. Biol. Chem.
25. Acs, G. et al. Manuscript in preparation.
26. DFP, diisopropyl fluorophosphate; NPGB, nitrophenyl-p-guanidobenzoate; TLCK, N-d-Tosyl-L-lysyl-chloromethyl ketone; TPCK, N-α-Tosyl phenylalanyl chloromethyl ketone; E-ACA, ϵ-aminocaproate

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27. Kelyalides, N.A. Chemical Properties of Basement Membranes, Int. Rev. Exp. Pathology 10: 1 (1971).

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The department of chemical biology contains all of the facilities necessary for the successful completion of this project. These include cold rooms, low temperature freezers, scintillation counters, tissue culture and media preparation facilities, incubators, spectrophotometers, etc. All of these facilities will be available for this study. Requests for equipment which would duplicate existing facilities have been made only for those items which are presently used to full capacity. The addition of those pieces of equipment requested will allow the proposed research to be carried out in an efficient and independent manner.

11. Additional facilities required:

The request for a biohazard containment hood is made because much of the proposed work will be with primary human tissue where the introduction of human pathogens into the laboratory is possible. At the present time the department does not have a containment hood for tissue culture work.

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

12.

Daniel B. Rifkin

Assistant Professor, The Rockefeller University Born: REDACTED

Nationality: REDACTED

Education:

Princeton University	A.B. REDACTED	Biology
The Rockefeller University	Ph.D.	Biochemistry
The Rockefeller University	Postdoctoral REDACTED	Biochemistry

Honors:

Jane Coffin Childs Memorial Fund Fellow	1968-1970
American Cancer Society Faculty Research Awardee	1972-1974

Major Research Interests:

Biochemistry, cell transformation, cell biology, tissue culture

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As a graduate student, I studied the protein and physical chemistry of several animal hemoglobins. This research was done under the supervision of Drs. William Konigsberg and Lyman Craig. Many of the techniques employed in the characterization of these proteins will be applicable to the study of the lung protease.

For the past five years I have studied the biochemistry of Rous sarcoma virus and the differences between normal and transformed cells. As a postdoctoral fellow in the laboratory of Dr. Edward Reich, I studied the lytic action of drugs which specifically kill transformed cells, the phospholipids of Rous sarcoma virus membranes as well as membranes from normal and transformed chick embryo cells, the effects of drugs which affect cellular RNA synthesis, and the organization of the proteins in Rous sarcoma virus. Since my appointment as assistant professor, I have developed a method for labeling proteins on the external membranes of vesicular structures. More recently I have been involved with the characterization of a novel proteolytic activity associated with malignantly transformed cells. This research has given me the tissue culture and biochemical skills necessary for the successful completion of this project.

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12.

Lloyd Howard Waxman

Date of birth: REDACTED

Place: REDACTED

Marital status: REDACTED

Social security #:

Education:

Temple University (Summa cum laude)	A.B.	Physics
Temple University (NSF Traineeship in Physics)	M.A.	Physics
Harvard University (NIH Traineeship in Biophysics)	Ph.D.	Biophysics

REDACTED

Teaching Appointments:

Teaching fellow in Biology	1/69-6/69
Teaching fellow in Biochemistry	9/69-1/70
Teaching fellow in Biochemistry	9/70-1/71
Teaching fellow in Biochemistry	9/71-1/72

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For the past four years I have been studying the oxygen-carrying proteins found in invertebrates. These proteins, which contain either copper or heme, generally have molecular weights in the millions, and are characterized by the high degree of cooperativity they exhibit upon binding oxygen. Consequently, they are of interest as models of protein-protein interaction over long distances, such as that which might be found in nerve conduction or on cell surfaces. The heme-containing pigments are composed of dimers which are held together by disulfide bonds and which contain one site for binding oxygen. The copper-containing proteins have polypeptide chains of molecular weight 3×10^5, which places them among largest ever described. Each chain contains six sites for binding oxygen, and it appears that it has been duplicated in identical repeating units of 5×10^4.

This project, which has some interesting genetic and evolutionary implications, has given me a great deal of experience in the purification and characterization of proteins and peptides, including a substantial amount of work with glycoproteins. More recently, because the main thrust in our laboratory has centered on membranes and membrane proteins, I have been using some of these techniques coupled with the use of detergents, to isolate various components of the erythrocyte and lobster nerve membrane.

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12. (continued)
L. Waxman

My present research interests are directed toward using techniques in protein, membrane, and carbohydrate chemistry to help characterize lesions in diseases which may involve the function of proteases or membrane proteins.

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14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Daniel B. Rifkin	50	-----
Lloyd Waxman	100	REDACTED
Technical		
John O'Malley	50	REDACTED
Staff Benefits	-	
Sub-Total for A		
B. Consumable supplies (by major categories)		
tissue culture supplies		3,000
radioactive isotopes		2,000
chemicals		1,000
glassware		1,000
Sub-Total for B		7,000
C. Other expenses (itemize)		
animal costs		500
publication costs		500
illustration costs		500
travel		500
Sub-Total for C		2,000
Running Total of A + B + C		26,328
D. Permanent equipment (itemize)		
Baker Biohazard Containment		4,000
Microscope	-	1,500
Water bath		300
Sub-Total for D		5,800
E		3,949
Total request		36,077

E. Indirect costs (15% of A+B+C) (15% of 26,328)

15. Estimated future requirements: 5.5% increase 1 year

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	7,385	2,000	--	3,969	31,635
Year 3	R	7,791	2,000	1,000	4,170	34,247

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16. Other sources of financial support.
List financial support from all sources, including own institution, for this and related research projects

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Surface Proteins of Normal and Transformed Cells	NIH CA-13138	92,153	1/1/72-12/31/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Renewal of above grant in 1974			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Daniel B. Rifkin

Signature Daniel B Rifkin Date 7/17/73

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Area Code Number Extension

Checks payable to

Rockefeller University

Mailing address for checks

c/o Miss Margery Pedersen
Manager of Accounting Services
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New York, New York 10021

Responsible officer of institution

Typed Name Dr. Frederick Seitz

Title President

Signature F. Seitz Date 7/24/73

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